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Application of an experimental design in frying oils degradation by *Shewanella*

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Aplicação de design experimental na degradação de óleos de fritura por *Shewanella.* Em Manaus, cerca 96% do óleo de fritura é descartado no meio ambiente sem tratamento, o que acarreta na poluição dos rios e solos, além de entupimento de encanamentos. A falta de políticas de coleta destes resíduos e a pouca atratividade dos produtos gerados a partir do mesmo (sabão, ração, etc), com exceção do biodiesel, requisita outras estratégias de biorremediação dos locais afetados. Visando isso, a *Shewanella putrefaciens* foi submetida a uma modelagem experimental, em um estudo preliminar, para a avaliação da adaptação em concentrações diferentes de óleos de fritura e em diferentes faixas de pH e de temperatura, uma vez que possui vias metabólicas capazes de aproveitar e degradar componentes desses óleos. Obteve-se um modelo com explicação de 82,4% com melhores faixas de temperatura entre 30-35 °C, pH 4-6 e concentração de 0,5-4% de óleo com uma taxa de degradação entre 75-85% do óleo.

Palavras-chaves: *Shewanella putrefaciens*; Superfície de resposta; óleo residual; glicose;

Abstract

In Manaus, about 96% of frying oil is discarded in the environment without treatment, which causes pollution of rivers and soils, as well as clogging of pipes. The lack of policies to collect these residues and the low attractiveness of the products generated from it (soap, feed, etc.), with the exception of biodiesel, require other bioremediation strategies of the affected sites. Aiming at this, *Shewanella putrefaciens* was subjected to an experimental modeling in a preliminary study for the adaptation evaluation at different concentrations of frying oils and in different pH and temperature ranges, since it has metabolic pathways capable of utilize and degrade components of these oils. A model with an explanation of 82.4% was obtained with better temperature ranges between 30-35°C, pH 4-6 and concentration of 10% oil with a degradation rate between 75-85% of the oil.

Key-words: *Shewanella putrefaciens*; response surface; residual oil; glucose.

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1. Introduction

Vegetable oils are composed of lipids found in vegetable tissues, most commonly extracted from seeds or grains, containing a concentration of 95% triacylglycerols (glycerol esters) and to a lesser extent monoacylglycerol, diacylglycerol, free fatty acids, vitamin E, proteins and sterols (DEL RÉ; JORGE, 2006; CORSINI; JORGE, 2008; LEHTINEN et al., 2017).

The world market, in 2016, consumed about 129 million tons of oil in the food sector, being reserved to Brazil an estimated 7 million tons. In Brazil, there are few public and private policy actions promoting the collection and awareness of the waste generated after the consummation, which affects the ecosystem and causes clogging in the pipelines. In Manaus, about 96% of the oil consumed is improperly discarded, with 51% corresponding to the discharge in drains (CASTRO, 2016; TOMAZI et al., 2014; SANTOS et al., 2015).

The process to eliminate this material and unclog the plumbing requires the use of toxic chemicals, thus creating a chain of treatments not only to remove grease fats, but also to remove those toxic products used. Because of that, the value of grease water treatment and sewage of a city can be increased by about 50% (ZUCATTO et al, 2013).

During the immersion frying process, oils are continuously exposed to several factors that lead to a wide variety of chemical reactions such as: hydrolysis, oxidation, triacylglycerol molecule polymerization, metal bonding, etc. (ALBUQUERQUE et al., 2014; CORSINI; JORGE, 2008; FREIRE et al., 2013; MACHADO et al., 2014).

In addition, these wastes possess toxins, after being exposed to high temperatures, such as high levels of peroxides and carbonates. Molecules such as 4-hydroxynonenal, an aldehyde product of the oxidation of linoleic acid, is one of the most commonly found substances (GUILLÉN; URIARTE, 2011; IZAKI et al., 1984; SEPPANEN; CSALLANY, 2002, 2004).

Since the current segments for these wastes (soap, detergent, animal feed, etc.) do not attract the market as much and there is no collection structure for biodiesel production, investments in other technologies such as microbial fuel cells (MFC) that can use these oils as input to the microorganisms and generate energy (FONSECA, 2018; GUABIROBA et al., 2014).

The gram negative bacteria from genus *Shewanella* is widely applied in MFC due to its ability to spin out electrons generated during the degradation of organic compounds to the environment. As it occurs, metal such as iron and oxides of manganese, sulfur and thiosulfate are reduced. Thus, such type of microorganism is prone to be applied widely in wastes containing high concentrations of metals, such as residual frying oils that bind to diverse metals, depending on the utensil and food used (BRETSCHGER et al., 2010; YIN; GAO, 2002).

In order to study describe the performance of *Shewanella putrefaciens* using frying oil as substrate and thus being a source of electric energy, we carry out laboratory essays using design of experimental (DOE).

2. Material and Methods

2.1 Microrganism

Shewanella putrefaciens, acquired from the André Tosello Foundation in 2013, by the DNA Laboratory of the Federal University of Amazonas (UFAM) was grown in liquid LB (Luria-Bertani) medium and incubated under 30°C and at 150 rpm.

2.2 Frying oil samples

About 1 liter of soybean oil used in the frying process was collected from residences, without selectivity of the food submitted to the process. The oil was sieved and filtered (UNIFIL 4-7µm) to remove solid waste from food and stored in oxygen-free (nitrogen bubbling) PTFE (Polytetrafluoroethylene) bottles.

2.3 Bacteria growth

To determine the cell growth time of *S. putrefaciens*, a pre-inoculum of the bacterium was made in LB medium for twelve hours at 30°C and 150 rpm. The pre-inoculum was diluted in sterile LB medium in order to obtain an initial 0.001absorbance (0 hour) for the growth curve and the inoculum was incubated at 30°C and 150 rpm.

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For spectrophotometer reading, 1 mL of the bacterial culture was removed every hour until reaching the cell decline phase and centrifuged for 10 min at 12,000 rpm. The supernatant was discarded and the pellet was suspended with 1 mL of PBS (phosphate buffered saline) 1X (4 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4) and read at 600 nm (Shimadzu UV-1800 Spectrophotometer).

2.4 Oil degradation measure

The oil degradation was calculated by comparison over the volume (µL) that was inoculated and what was recovery in the end of the each experiment (Equation 1).

Equation 1: $Oil\ degradation\ (\%) = \frac{oil\ recovery \times 100}{oil\ inoculated}$

2.5 Experimental design

This is a preliminary study to determine which variables are significant and the respective levels that can be applied in the conditioning of *S. putrefaciens* in microbial fuel cells (MFC) aiming at the maximum degradation of these oils in anaerobic environment and low bacteria growth, since a high cellular concentration can lead to problems in the electrochemical balance of the MFC and compromise the energy generation.

The tests proceeded using liquid medium M9 (42.5 g/L $Na_2HPO_4.2H_2O$, 15 g/L KH_2PO_4, 2.5 g/L NaCl, 5 g/L NH_4Cl, 2 mL/L $MgSO_4$ 1 M and 100 µL/L $CaCl_2$ 1M) with frying oil as substrate in 10 mL threaded test tube (SAMBROOK; RUSSEL 2001 modified) under the conditions indicated in the experimental design generated in the Statistica v.12.

The oil did not mix with the M9 medium and it was not in the interest of the study to add an emulsifier, so an agitation of 110 rpm was set to increase the bacterial surface contact with the oil in the tube area.

The metabolic pathways for the degradation of fatty acids are not a priority, so a period of 48 hours was fixed to guarantee the visualization of more expressive results.

Using the Central Composite (2^3) experimental design, it was observed the influence of some variables in different levels (-1.682, -1, 0, +1 and +1.682), which these are temperature (25 – 40°C), pH (4.3 – 7.6) and oil concentration (1.5 – 18.5%) on the total volume of the medium (5 mL) in a period of 48 hours and agitation of 110 rpm (Table 1). To analyze at the program, it was obtained the degradation rates (%) of the oil as a response.

Table 1 – Factors used in the planning of the central composite with their respective levels.

Factors	Values		Levels obtained in statistical planning				
	Minimum	Maximum					
	-1.682	+1.682	-1.682	-1	0	+1	+1.682
Temperature (°C)	25	40	25	28	32.5	37	40
pH	4.3	7.6	4.3	5	6	7	7.6
Oil concentration (%)	1.5	18.5	1.5	5	10	15	18.5

In all, nineteen (19) experiments were performed randomly with the five repetitions only for the central point (pH 6, 10% oil and 32.5°C). The chosen factors were select based on the microorganism mesophilic characteristics, the final oil pH (4.5-6) and the ability of the bacteria to adapt in different pH range.

In parallel, cell growth was compared with de frying oil degradation rates in each condition to investigate to assess the relationship between bacterial growth and oil degradation but was not used as response at central composite.

2.6Bacteria growth vs oil degradation

Before carrying out the experiments outlined in topic 2.5, control tests were performed using glucose, mineral oil and frying oil, where they constituted 10% of the

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medium (glucose 1M, 5% frying oil + 5% glucose, mineral oil and frying oil), in the ranges of pH and temperature indicated on the experimental model to ascertain the cell growth of the bacterium at 600 nm under the above conditions. The initial cell absorbance of all experiments was 0.001.

Also was done experiments without any carbon source, using only the M9 medium according to experimental model. To study the capacity of the medium to maintenance the cellular concentration, the initial cell absorbance was 0.2.

The spectrophotometer readings of all experiments were made according what was describe in item 2.3.

2.7Statistical analysis

The results were analyzed in Statistica12.0 for the generation of surface graphs and mathematical model, besides the analysis variance (ANOVA) with p = 0.05. In addition, the F-Test was also performed to ascertain the quality of the model generated.

2.8 Model efficiency

To verify the efficient of the mathematical model generated after the statistical analysis, was performed an experiment using randomly chosen factors (35°C, pH 4 and 15% of oil) during 48 hours at 110 rpm and compared the theoretical results with the actual results in triplicate.

3. Results and Discussion

The bacteria *Shewanella putrefaciens* has the β-oxidation pathway, responsible for the degradation of fatty acids, as well as the glycosidic (RODIONOV et al., 2011), which allows us to observe the formation of biomass, when applied to these nutrients or the combination of them culture medium (Figure 1).

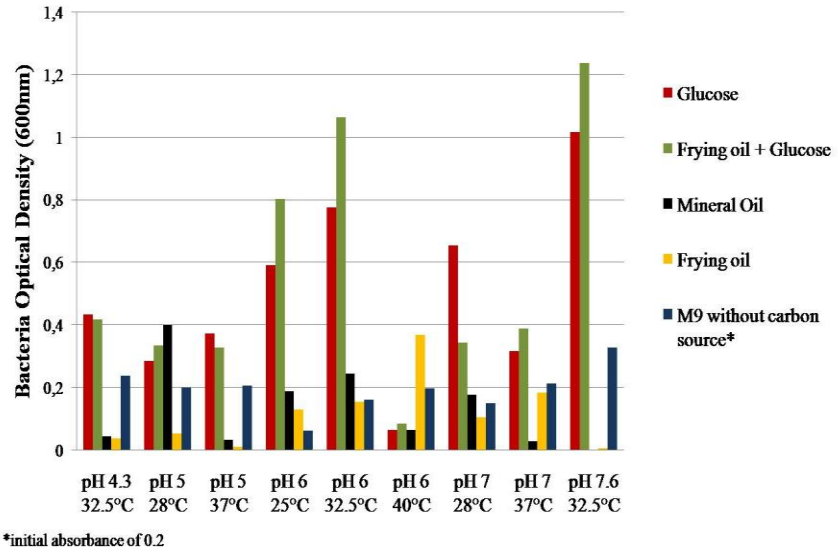

Figure 1 - Cell growth of *S. putrefaciens* using glucose, mineral oil, frying oil and a mix of frying oil and glucose in control groups at different temperature and pH ranges after 48 h of incubation.

It was not observed an expressive biomass formation using only frying oil in general, but in the presence of the combination of the residual oil with glucose the biomass is higher than using only glucose.

The bacteria *S. putrefaciens* has several electron receptors such as iron, chromium and manganese, which is an advantage for the process, because these metals are present in oils of domestic origin, such as the one used in this study. The presence of Fe ions collaborates with the biomass formation of *S. putrefaciens*, since its receptor is next to the electron transport chain (MYERS; MYERS, 1994; MYERS et al., 2000; WEBER et al., 2006; YIN; GAO, 2002).

Thus, the ease of glucose uptake combined with the presence of metal ions in the

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oil contributes to a prolonged maintenance of bacterial cell life.

No significant biomass concentration was observed having only residual oil as the carbon source at the end of the test, with a higher amount in mineral oil in most trials, a compound known to preserve bacterial cultures by reducing oxygen consumption by 10%, but which shows little efficacy for the preservation of the bacterium in question, since it did not maintain a constancy of the biomass, reaching its death in the last test (pH 7.6 and 32.5°C) (HARTSELL, 1953).

In general, the experiments using only M9 medium without any carbon source or preservative maintained the same initial cell concentration (0.2). Was observed a decrease in cell concentration was observed in the experiments at pH 7 and 28°C, pH 6 and 32.5°C and pH 6 and 25°C, especially in the latter with an absorbance of 0.064. Only on experiment at pH 7.6 and 32.5°C was detect an increase in cell concentration (0.329). To maintain the culture in M9 medium without carbon source is indicated use pH 5 between 28-37°C, according with the results.

The M9 medium was originally developed to give support to *Escherichia coli* and *Salmonella typhimurium* and has low osmolarity. It's possible that the medium needs to be supplemented with Fe and Cr ions to maintain the cell density in large ranges of pH and temperatures at anaerobiosis state (MYERS et al, 2000; NEIDHART et al, 1974; WEBER et al., 2006)

The LB medium has D-glucose as one of the compounds and is considerate as a rich medium to bacteria growth (Figure 2). If comparing the results obtained at Figure 1 (48 hours incubation) with the results on Figure 2 (37 hours incubation), the bacteria is on cell decline phase at experiments using glucose and frying oil + glucose. Final cell density optical correlating with the degradation of the oil in the conditions of experimental design (Table 2), it is observed that most of the tests at temperatures above 30°C has a cell much higher than at lower temperatures. On the other hand, the rate of degradation is higher between ranges of pH 6 - 7.6.

At Table 1, at 25 °C and 40 °C, even at pH 6, 27% and 42% oil degradation rate were observed, respectively. In this last one, the highest optical density of the assays was observed (Figure 1).

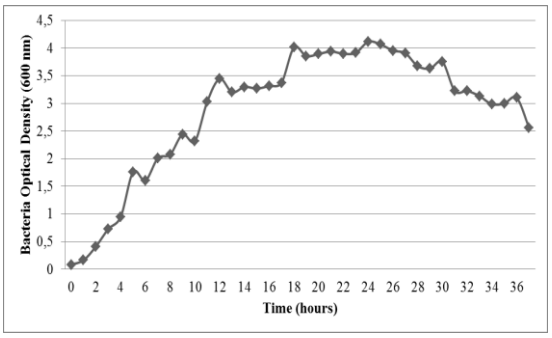

Figure 2 - Cell growth of S. putrefaciens in LB medium for 37 hours at 30°C and 150 rpm.

At different assays (2 and 7), was observed minimal (0.007) and half (0.155) of biomass observed at assay 15, but with 33% and 53,4%, respectively, of oil degradation, which indicates that cell growth is not linked necessarily to the degradation of the frying oil.

In Figure 3, it's shown the effects studied in the experimental design. The three factors (temperature, pH and oil concentration) show significance for the composition of the model.

The pH-oil interaction was negative, implying an antagonistic effect of both variables. Thus, to increase the response (oil degradation) using larger ranges of concentration of oil should be fixed at lower pH ranges.

The significance of the oil-pH interaction occurs by lipid oxidation that is increased in acidic pH (LEE; NEWMAN, 2003; LOVLEY; HOLMES; NEVIN, 2004) and it can be verified at Figure 4, which shows that, if the pH range is increased, lower is the oil degradation.

Based on the factors studied and their interactions, surface graphs were generated to determine the ideal conditions for frying oil degradation by *S. putrefaciens*, in addition to the formulation of a quadratic equation to model the system and analysis of variance (ANOVA) for to ascertain how much of the model was explained.

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Table 2 - Optical density of *Shewanella putrefaciens* and residual frying oil degradation rate after 48 hours under experimental planning test conditions.

Assay	Conditions	Bacteria Optical Density (600 nm)	Oil Degradation (%)
1	25°C, 10% of oil and pH 6	0.130	27
2	28°C, 5% of oil and pH 5	0.007	33
3	28°C, 15% of oil and pH 5	0.055	44
4	28°C, 5% of oil and pH 7	0.106	67
5	28°C, 15% of oil and pH 7	0.030	53
6	32.5°C, 10% of oil and pH 4.3	0.038	44
7	32.5°C, 10% of oil and pH 6	0.115	53
8	32.5°C, 10% of oil and pH 6	0.117	57
9	32.5°C, 10% of oil and pH 6	0.129	50
10	32.5°C, 10% of oil and pH 6	0.259	50
11	32.5°C, 10% of oil and pH 6	0.126	57
12	32.5°C, 1,5% of oil and pH 6	0.097	100
13	32.5°C, 18,5% of oil and pH 6	0.190	61
14	32.5°C, 10% of oil and pH 7.6	0.007	55
15	37°C, 5% of oil and pH 5	0.011	33
16	37°C, 15% of oil and pH 5	0.100	36
17	37°C, 5% of oil and pH 7	0.184	67
18	37°C, 15% of oil and pH 7	0.100	35
19	40°C, 10% of oil and pH 6	0.370	42

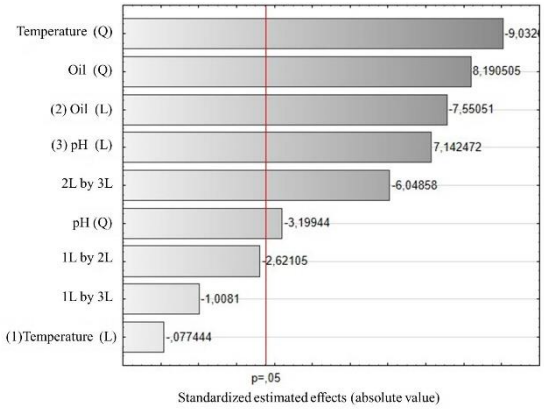

Figure 3 - Pareto diagram with the factors (y-axis) and effects estimation (x-axis) with p=0.05 based on the frying oil degradation data by *Shewanella putrefaciens*.

It was possible to explain about 82.4% of the phenomena that occurs during the oil degradation with the possibility to reach 98.9%, if corrected adjustments, according to the ANOVA.

The regression represents what occurs in proposed model and the residuals are the phenomena that were left out of the model. Keeping that in mind, in order to validate the experimental modeling, the F test (Equation 2) was performed using the F tabulated (4.45)

Equation 2:

$$\frac{\text{Regression Q.A.}}{\text{Residue Q.A.}} = 79.55 > 4.45 = F_{1,17}$$

As a result, it is shown that the regression is significant, there is a relation between the studied factors to compose the quadratic model and few external factors could affect the system.

Since there are adjustments and errors pointed out by ANOVA, another F test was made to find out if there is any way to improve the study model (Equation 3) using the F tabulated (3.41)

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Equation 3:

$$\frac{\text{Lack of fit Q.A.}}{\text{Pure error Q.A.}} = 72.96 > 3.41 = F_{3,13}$$

As described in Equation 2, the quadratic average (Q.A.) of the lack of fit is higher than the Q. A. of experimental error, which demonstrates that it is possible to improve the model.

As result of this regression, we obtained a quadratic equation (Equation 4) that models the influence of pH, temperature and oil concentration on the medium in the rate of oil degradation.

Equation 4: $\text{Degradation (\%)} = 53.652 + 6.841\text{pH} - 7.133\text{oil} - 0{,}737\text{temp} - 3.125\text{pH}^2 + 7{,}63\text{oil}^2 - 8.691\text{temp}^2 - 7{,}5\text{pHoil} - 1{,}25\text{pHtemp} - 3{,}25\ \text{oil.temp}$

Equation 4 is coded, so to use it one must use the levels (-1, +1, etc.) corresponding to the values of interest to apply in the model.

According to Pareto diagram (Figure 3), pH (L) has a positive effect, which indicates that the increase in pH increases the rate of degradation, but since pH (Q) is negative, it is only possible to improve the response to a certain level. Using the graph below (Figure 4), it is possible to check the conditions for a degradation of oil in different pH ranges.

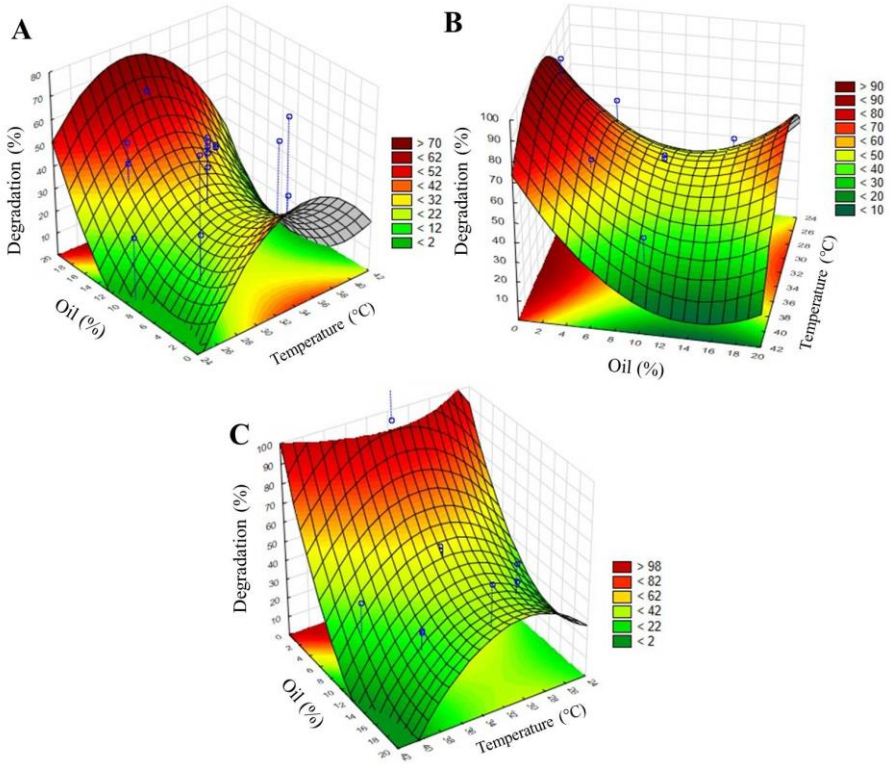

Figure 4 -Response surface obtained from the quadratic equation obtained in the study of the influence of temperature and oil concentration factors, with residual oil degradation by *Shewanella putrefaciens* as a response in the condition of (A) pH 4, (B) pH 6 and (C) pH 8.

As the pH increases, the maximum concentration of oil that can be applied in the process to obtain a high degradation rate becomes smaller and smaller. At pH 4 and 33-35 ° C, 20% oil can be employed to achieve ± 75% degradation thereof. At the same temperature and pH 6 and 8 conditions, the degradation drops to ± 65% and ± 40% respectively.

In Figure 5, the analysis points are the ratio of oil concentration and pH in different temperature ranges.

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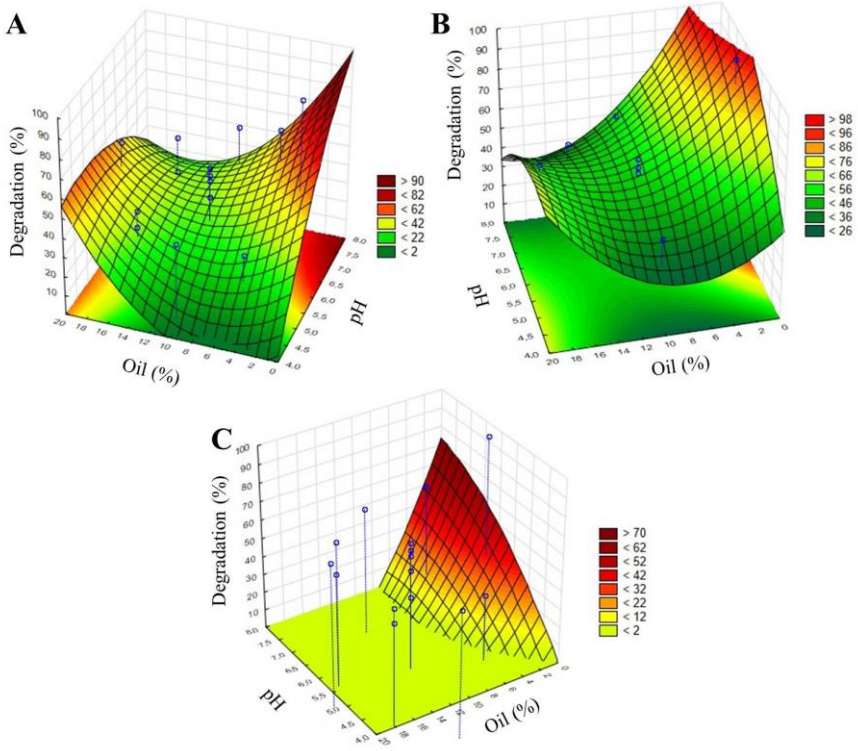

Figure 5 -Response surface obtained from the quadratic equation obtained in the study of the influence of pH factors and oil concentration with residual oil degradation by *Shewanella putrefaciens* as response in the condition of (A) 25°C, (B) 35°C and (C) 45°C.

At 25°C, 62% of the oil may be degraded at a concentration of 20% in a range of pH 4-6. Under other conditions, the oil concentration is low or the degradation rate does not exceed 30%.

At 35°C, 66-75% of the oil can be degraded at an oil concentration and pH range equal to 25°C, but in a system with 10% oil, it is possible to reach 85% degradation. At 45°C, the pH range is negligible and it is possible to degrade only small concentrations of oil.

High temperatures (above 40 °C) are detrimental to the metabolism of the bacteria, since it is mesophilic, and can't be applied for the degradation of frying oil, even that were cases that still survives at this temperature range with different carbon source. However, in a range between 30 – 35 °C, the metabolism is stimulated to catabolize the fatty acids of the frying oil (HAN; GRALNICK, 2007).

In the final data treatment, we obtain that to optimize the oil degradation rate, the best temperature ranges are between 30 – 35°C, pH 4 – 6 and 0,5 – 4% oil in the residual oil degradation model, but with the intention of using higher concentrations of oil (15-20%), it is recommended to use pH 4 and a temperature of 30-32°C.

After using the Equation 4 to predict the degradation at pH 4, 35°C and with 15% of oil, the theoretical result was 39,52%, but the actual results was 34,66±1%. Early, was pointed that de model need adjustments, but still can be used to give an estimative.

4. Conclusion

The study was conducted on a preliminary basis to observe the adaptation of the bacterium to frying oils on a test tube scale, so that it is possible to change the mathematical model by increasing the scale, making a new study necessary in this case. The application of the model for bioremediation of rivers and soils, as well as in microbial fuel cells, requires more research with the possibility of generating new models for each case.

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6. Divulgation

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